Archives of Razi Institute, Vol. 64, No. 1, June (2008) 23-28

Proteases Detection of in vitro culture of midgut cells from *Hyalomma anatolicum anatolicum* (Acari: Ixodidae)

Gholam Hossein Goudarzi [*1], B., Abdigoudarzi[2], M., Piriaii [4]M., Bagherian[3], R., Goudarzi[5], H., Karimi[2], GH.

1. Biotechnology Department, Razi Vaccine and Serum Research Institute, Karaj, Iran
2. Parasitology Department, Razi Vaccine and Serum Research Institute, Karaj, Iran
3. Biochemistry Department, Tehran University of Medical Sciences, Tehran, Iran
4. Department of Zoology, Payame Nour University of Isfahan, Isfahan, Iran
5. Department of Biochemistry, Payame Nour University of Tehran, Iran

Received 03 Nov 2007; accepted 20 Apr 2008

ABSTRACT

Proteases play a key role in protein digestion in ticks and other haematophagous insects. Our understanding of blood meal digestion in digestive system of ticks can be very useful for better understanding of basic rules for control of ticks. Cells of the midgut endocytose blood components. Blood proteins uptake by midgut cells, suggesting the presence of proteases in the midgut cells. In this study, proteases which may be present in midgut cells of engorged female ticks (*Hyalomma anatolicum anatolicum*) have been studied. The midgut tissue from tick was dissociated to cells. The cells placed in a culture plate with special medium. Then cell extract was obtained from the in vitro cultured midgut cells. Then cells were rinsed in hypotonic solution. The cellular suspension was centrifuged and supernatant removed from cell membrane plette. Cell membrane proteins were isolated by Solution 2% of Triton X-100. Proteases assay performed by specific substrates for cytoplasmic proteins and Cell membrane bound proteins. Then, enzyme like trypsin, enzyme like chymotrypsin, carboxypeptidase B, cathepsin C, cathepsin B and cathepsin D were detected. Our results show that enzyme like trypsin is a membrane bound protein, but carboxypeptidase B, cathepsin C, cathepsin B and cathepsin D are included in the cytoplasm of mid-gut cells. There is no enzyme like chymotrypsin in mid-gut cells of tick.

Keywords: *Hyalomma*, tick, protease, detection, assay

INTRODUCTION

Ticks are blood-feeding ectoparasites that transmit a wide variety of pathogens including arboviruses, rickettsiae, spirochetes and protozoa to humans and domestic animals (Sojka *et al* 2007). One of the most economically important species of tick which members are distributed in different geographical regions in Iran, Middle East, and some parts of Asia is *Hyalomma anatolicum anatolicum* (Abdigoudarzi 2004). There are studies to develop a vaccine using midgut antigens from *Hyalomma anatolicum anatolicum*, but little or no progress has been made. As obligate blood feeders, one possible strategy to retard disease transmission is disruption of the parasite's ability to digest host proteins. However, the constituent proteinase in the parasite gut and their potential interplay in the digestion of the blood

*Author for correspondence. E-mail: B-Goudarzi@rvsri.ir

meal are poorly understood. But obviously digestion of host proteins, including hemoglobin, by gut-associated proteinase and peptidase is an essential event for ticks that provides energy and nutrition for parasite molting and vitellogenesis(Grandjean 1984). Also, hemoglobin hydrolysis derives antimicrobial peptides vital for the survival of both the hard and soft ticks *Boophilus microplus* (Fogaca *et al* 1999) and *Ornithodoros moubata* (Nakajima *et al* 2003), respectively. Therefore, preventing the parasite's ability to digest host proteins might provide a useful strategy to interfere with disease transmission by decreasing the fecundity of ticks. There are some ideas that in contrast to insect blood feeders (Terra & Ferreira 1994), the contents of the midgut of tick are believed to be free of extracellular digestive enzymes. Rather, host blood proteins are gradually endocytosed with digestion occurring within gut cells (Sonenshine 1991). It is obvious that proteases are present in most midgut cells of ticks. They play a key role in protein digestion in ticks and other haematophagous insects. Our understanding of blood meal digestion in digestive system of ticks can be very useful for better understanding of basic rules for control of ticks. The cattle tick ingests 100 times of its own mass with blood. Cells of the midgut endocytose blood components. Blood proteins uptake by midgut cells, suggesting the presence of proteases in the midgut cells.

After blood feeding by ticks, blood should be digested by Enzymes (proteases). If the ingested blood remains intact (indigested) then problems could be arise for ticks. Indigested blood could block the midgut and may result to tick death. There will be IgG production after injection of proteases taken from tick to the host. This produced IgG could interfere with normal blood digestion in the midgut of ticks, or even may lead to the death of the tick. One other advantage of targeting serine proteinases as candidate vaccine antigens is the fact that they are generally secreted in the extra cellular environment and hence they are likely to be exposed to host antibodies (Vaughan & Azad 1988; Ben-Yakir 1989; Allingham *et al* 1992). Thus far, both cysteine and aspartic peptidases are believed to contribute to hemoglobinolysis, however, few reports characterizing individual peptidases exist (Sojka *et al* 2008), (Ferreira *et al* 1994).

MATERIALS AND METHODS

Isolation of midgut of *Hyalomma*. External surface of Laboratory bred ticks which were cultured by the Department of parasitology, have been washed and ticks were suspended in chlorohexidine 0.2%, and ethanol 70%; and finally in three changes of sterile distilled water. The washed ticks were dissected and their midguts were separated and properly cleaned from the blood meal. (Akhtar and Hayat 2001). Then, cleaned midgut were washed and suspended in sterile PBS containing antibiotic and antifungal.

Midgut cell culture. The midgut tissues were dissociated into cells by collagenase buffer and sterilized tissue grinder. Cells were collected using centrifugation and washed three times in the Hanks balanced salt solutions. The cell suspensions placed in a 12-well culture plate with TC-100 medium which Supplemented with heat inactivated (56 ºC for 30 minutes) 5% FCS, antibiotics (Penicillin 100 IU/mL, Streptomycin 100 µg/mL and Nistatin 50 IU/mL) and was maintained at 28ºC (Akhtar *et al*., 1992). After 2 days, the cells adherents to the well's surface were harvested by a rubber policeman and media were transferred to the sterile centrifuge tubes. The wells were rinsed two to three times with 5 mL sterile phosphate buffered saline (PH 7.2) and added to the tubes containing harvested cells in media. Finally, pooled cell suspension was centrifuged (5000 rpm/30 minutes/4 ºC) and the supernatant was removed. Cells from sediment were repeatedly washed in fresh phosphate buffered saline (PBS) by centrifugation as described above. It

is to be noted that cells were counted before and after cell culture on Neobar slide.

Preparation of Proteins.Cell pellet was collected and cells were resuspended in minimum volume of PBS, 2% triton X-100 (1:1) then it was homogenized shaking it for 1minute. Then it was dialyzed for 2 hours against distilled water and stored at -20 ºC. Extracted proteins from homogenized cell suspension were used for protease assay. Protein concentration in the extracted material determined according to the method of Lowry *et al*. **(**Lowry 1951). Crystalline bovine serum albumin was used as a standard.

Measurement of enzyme activity. In this study, Activity of proteinases was determined spectrophotometrically.

Determination of Trypsin like enzyme activity. It was measured using BAPNA as substrate according to the method of Erlanger (Erlanger et al 1961).

Determination of Chymotrypsin like enzyme activity. Activity of Chymotrypsin like enzyme was measured according to the method of Rick, W. (Rick 1974). In this method Na-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) was used as substrate.

Determination of Carboxypeptidase activity. Carboxypeptidase activity was determined from the initial rates of hydrolysis of Hippuryl-L-Arginine in 25mMTris-HCl buffer with 100mM Sodium Chloride, pH 7.65 at 25 ^{o}C, following the procedure of Folk (Folk *et al* 1960).

Determination of cathepsin B activity. It was performed according to Bajkowski method (Bajkowski & Frankfater 1975). In this method using Ná-CBZ-L-Lysine p-Nitrophenyl Ester as substrat.. Ná-CBZ-L-Lysine p-Nitrophenyl Ester is a specific substrate that releases p-Nitrophenol when digested with cathepsin B. was used.

Determination of cathepsin C activity: Active cathepsin C (dipeptidyl aminopeptidase I) was confirmed according to method of (Planta & Gruber 1963). So that cathepsin C activity was measured spectrophotometrically using Gly-Phe-p-nitroanilide (Sigma) as substrate.

Determination of cathepsin D activity- Activity of cathepsin D was measured by its ability to cleave hemoglobin as substrate according to Barrett, A.J. method (Barrett 1970). In this method, in condition 100 mM Formic Acid Buffer, pH 3.3 at 37 °C, hemoglobin cleaved by cathepsin D to TCA-soluble peptides. Then TCA, 5 % was added at two fold of the volume of reaction mixture and it was incubated for 10 minutes at 37°C. The solution was filtered through a 0.2 mm syringe filter. The solution was transferred to suitable cuvettes and the absorbance was read at 280nm for both the Test and Blank solutions using a suitable spectrophotometer. One unit of enzyme is equivalent to 1 unit of addition of absorbency which produce 1 unit increase in 280 nm in 30 minutes at pH 3.3 at 37 °C measured as TCA-soluble products using acid denatured hemoglobin as substrate (1 cm light path).

RESULTS

Some of midgut cells of tick are seen in figure 1. In this figure, there are two types of cells in culture medium and they could be easily seen. Data from cell count and viability test studies are seen in table 1. Numbers of cells before and after cell culture were 42.2×10^6 and 35×10^6 cells respectively and 82 percent of cells showed to be vital.

Results from total protein assay on homogenized cell suspension were done. The protein concentration was 39.9 mg for homogenized cell suspension Standard protein curve was drawn after analyzing data from different concentration of albumin and their related absorbencies (figure 2).

Results of assay for proteases show that there are enzymes like trypsin, carboxypeptidase B, cathepsin B, cathepsin C, cathepsin D, but chymotrypsin like enzyme doesn't present in midgut cells of tick. As it is shown in table 2 specific activity for trypsin, carboxypeptidase B, cathepsin B, cathepsin C,

cathepsin D, like enzymes are 6.58, 5.62, 3.16, 2.41, 6.77 respectively.

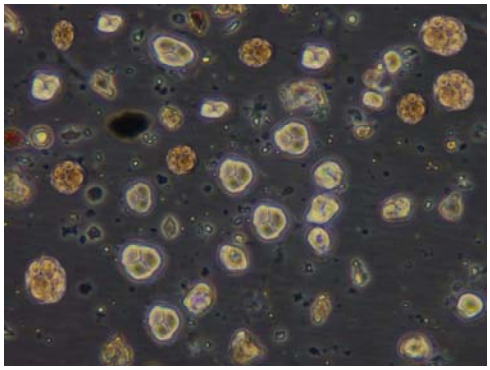

Figure 1. Two types of cells from culture of midgut cells of tick in TC-100 medium after 48 hours (arrow).

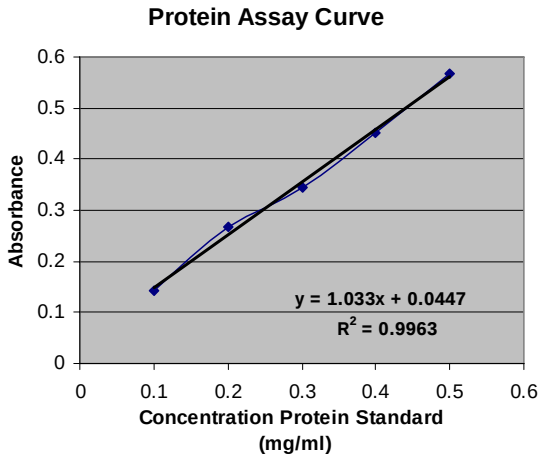

Figure 2. Standard protein curve using the protein concentrations and their related absorbencies.

DISCUSSION

The economic impact of tick infestations is enormous worldwide. In 1984, the United Nation Food and Agriculture Organization (FAO) estimated the global cost of Ixodidae tick infestations to be US$7.0 billion annually (Harrow *et al* 1991). The one-host tick *Boophilus microplus* is reported to have caused losses of more than US$10 billion and US$10 million annually in South America and Australia, respectively (Radostits *et al* 2000). Apart from the direct effects of tick infestations on animal production and productivity, ticks are inevitably efficient vectors of many pathogens—protozoa, viruses, bacteria, and rickettsiae to man and domestic animals (Walker *et al* 1978) and (Radostits *et al* 2000). Then study of ticks is necessary and useful for their control.

Table 1. Data from cell count and viability test before and after cell culture of midgut cells from tick (*Hyalomma anatolicum anatolicum*)

step	Volume media of cell culture	N. of cell in 1 ml	Total cell count	viability
Before cell culture	10	42.2×10^4	42.2×10^6	-
After cell culture	10	35×10^4	35×10^6	82

It is to be said that midgut cells of an engorged tick have proteins which their origin is from blood taken from host because blood digestion in ticks is intracellular in midgut cells (Sonenshine 1970).

Table2. Total activity and specific activity of extracted enzymes of midgut cells from tick (*Hyalomma anatolicum anatolicum*)

Type of Enzyme	Specific Activity	Total Activity
Trypsin Like Enzymes	6.58	262.75
Chymotrypsin Like Enzymes	0	0
Carboxypeptidase B Like Enzymes	5.62	224.45
Cathepsin B Like Enzymes	3.16	126.2
Cathepsin C Like Enzymes	2.41	95.15
Cathepsin D Like Enzymes	6.77	270.075

It is clear that culturing the cells from midgut of tick is very important because when cells are cultured there will be enough time for cells to get rid of bloods and proteins originated from host blood.

Then one could be able to diagnose original proteins from cells of the midgut of tick. Also we can determine situation of proteases and other proteins when we have intact midgut cells. Results got from viability tests showed that 82% of cells were viable after 48 hours and results from cell counts before and after cell culture showed that 7.2 million cells were died. There may be some WBC from host blood cells that they could be counted as cells from midgut of ticks before cell culture. Culture medium used in this study is specific culture medium for insects cells, so, cells from host disappeared and there is no CO2 when culturing the cells. Protein digestion in midgut of ticks is accomplished in acidic condition and lysosomal enzymes are engaged in this process too. There is report that blood digestion in midgut cells is incorporated in acidic and alkaline conditions. Different catepsin(B, C, D) enzymes and carboxypeptidase B also is reported (Lehane & Billingsley 1996)(Sonenshine 1991). In our study different trypsin like enzymes and carboxypeptidase B like enzymes in alkaline medium and different Catepsin (B, C and D) in acidic medium are reported. There are limited studies about different enzymes from midgut of ticks and little reports about trypsin like enzymes are documented. There may be studies focused on homogenates from midgut of ticks, and then they failed to find any trypsin like enzymes in their studies. The antiproteases in blood from host may interfere and they could inactivate trypsin like enzymes and other serine proteases, so, there are no reports of these enzymes. In our study when the midgut cells were allowed to grow in culture medium, then there have been enough time to overcome to the related problem and there was enough time for digestion of different peptides originated from host and those antiproteases will be destroyed and different proteases could be confirmed.

References

Abdigoudarzi, M. (2004). Review of Ticks (Acari: Ixodidae) and redescription of *Hyalomma* and *Rhipicephalus* genera by RAPD-PCR in Iran. *Thesis in partial fulfillment of the doctoral degree in entomology. Tarbiat Modares University.*

Akhtar, M. and Hayat C.S., (2001). Protective efficacy of subunit antigens isolated from in vitro midgut cells of *Boophilus microplus*. *Pakistan Journal of Biological Sciences* 4(9): 1165-1169.

Allingham, P.G., Kerlin R.L., Tellam R.L., Briscoe S. and Standfast H.A. (1992). Passage of host immunoglobulin across the mid-gut epithelium into the haemolymph of blood-fed buffalo flies (*Heamatobia irritans exigua*). *Journal of Insect Physiology* 38: 9–17.

Bajkowski, A.S. and Frankfater, A. (1975). Specific spectrophotometric assays for cathepsin B1. *Analytical Biochemistry* 68: 119-127

Barrett, A.J. (1970). Determination of cathepsin D activity. *Biochemical Journal* 117: 601-607.

Ben-Yakir D. (1989). Quantitative studies of host immunoglobulin in the haemolymph of ticks (Acari). *Journal of Medical Entomology* 26: 243–246.

Erlanger, B. F., Kokowsky, N. and Cohen, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics 95:271*-8.

Ferreira, C., Capella, A.N., Sitnik, R., Terra, W.R. (1994). Digestive enzymes in midgut cells, endo- and ectoperithrophic contents, and peritrophic membranes of *Spodoptera frugiperda* (Lepidoptera) larvae. *Archives of Insect Biochemistry Physiology* 26: 299–313.

Fogaca, A.C., da Silva, P.I. Jr., Miranda, M.T., Bianchi, A.G., Miranda, A., Ribolla, P.E. and Daffre, S. (1999). Antimicrobial activity of a bovine hemoglobin fragment in the tick *Boophilus microplus. Journal of Biological Chemistry* 274: 25330–25334.

Folk, J.E., Piez, K.A., Carroll, W.R. and Glander, J.A. (1960). Carboxypeptidase B: purification and characterization of the porcine enzyme. *Journal of Biological Chemistry* 235: 2272-2277

Franta Z., M. Horn, D.Sojka1, M. Mares, and P.Kopácek Mapping of hemoglobin proteolysis in the hard tick *Ixodes ricinus. Journal of Insect Science:* Volume 6 Article 4

Grandjean, O. (1984). Blood digestion in *Ornithodoros moubata* Murray sensu stricto Walton (Ixodoidea: Argasidae) females. I. Biochemical changes in the midgut lumen and ultrastructure of the midgut cells, related to intracellular digestion. *Acarologia journal* 25: 147-165.

Harrow, I. D., Gration K. and Evans, N. (1991) Neurobiology of arthropod parasites. Journal of *Parasitology* 102:559-569.

Lehane, M. J., Billingsley P. F. (1996). *Biology of the Insect Midgut*. Published by Springer, 486 pages.

Lowry, O.H., Rosbrough N.J., Farr A.L. and Randall R.J. (1951). protein measurement with the Folin phenol reagent *Journal of Biological Chemistry* 193: 265-275.

Nakajima Y.; Ogihara K.; Taylor D. and Yamakawa M. (2003). Antibacterial hemoglobin fragments from the midgut of the soft tick, *Ornithodoros moubata* (Acari: Argasidae*). Journal of medical entomology* 40(1): 78-81.

Planta, R.J. and Gruber, M. (1963) A simple estimation of cathepsin C using a new chromogenic substrate. *Analytical Biochemistry* 5: 360-362.

Radostits, M.O., Gay, C.C., Blood, D.C. and Hinchcliff, K.W. (2000). *Veterinary medicine: a textbook of the diseases of cattle, sheep, pigs, goats and horses.* pp. 1401-1405. Saunders, London

Rick, W. (1974) *Methods of Enzymatic Analysis*, 2nd ed.,Volume II, pp 1009-1012, Academic Press Inc., New York, NYOOSTRA, GM, NS.Sojka, D., Ondrej, H. *et al* (2007). IrAE – An asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. *International Journal for Parasitology* 37: 713–724

Sojka, D., Zdenek F., Martin H., Ondrej H., Conor, R., Caffrey, M. M. and Kopacek, P. (2008). Profiling of proteolytic enzymes in the gut of the tick *Ixodes ricinus* reveals an evolutionarily conserved network of aspartic and cysteine peptidases *Parasite Vectors* 1(1): 7.

Sonenshine, D.E. (1991). *Biology of ticks*. Vol II, 465p. Oxford University Press, New York.

Terra W.R. and Ferreira C. (1994). Insect digestive enzymes, properties compartmentalization and function. *Comparative Biochemistry Physiology* 109: 1-62

Vaughan J.A. and Azad A.F. (1988). Passage of host immunoglobulin G from blood meal into haemolymph of selected mosquito species (Diptera: culicidae). *Journal of Medical Entomology* 25: 472–474.

Walker, J.B., Mehlitz, D. and Jones, G.E. (1978). Effects of ticks infestations. Notes on the ticks of Botswana. *German Agency for Technical Cooperation* (GTZ) pp. 12-14 Eschborn Germany.